Endogenous cardiac Ca^{2+} channels do not overcome the E–C coupling defect in immortalized dysgenic muscle cells: evidence for a missing link

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Received 26 May 1995; revised version received 7 June 1995

Abstract **The expression of subunit genes of the Ca^{2+} channel complex was studied in differentiating, immortalized mouse *mdg* cells. These cells expressed α_1 and α_2/δ transcripts of the skeletal muscle Ca^{2+} channel genes, a cardiac Ca^{2+} channel α_1 subunit gene and several known transcript variants of skeletal, cardiac and brain β genes. The *mdg* mutation is retained in the $129DA_3$ cell line and occurs exclusively at nucleotide position 4010 in the skeletal α_1 transcript in which a cytosine residue is deleted. In early stages of differentiation and fusion, Ba^{2+} currents were detected in dysgenic myotubes the same as the cardiac L-type Ca^{2+} channel. These data provide specific structural evidence [Chaudhari, N. (1992) J. Biol. Chem. 267, 25636–25639] for the major genetic defect in mouse muscular dysgenesis and show a change in the expression levels of α_{1S} and α_{1C}. The upregulation of the expression of α_{1C} results in functional Ca^{2+} channel activity, however, presumably not sufficient for excitation–contraction coupling.**

Key words: Muscular dysgenesis; Calcium channel; α_1 Subunit; Skeletal; Cardiac; Point mutation; Mouse

1. Introduction

Muscular dysgenesis (*mdg*), a spontaneous, lethal mutation in mice [1], was found to be transmitted as a single autosomal, recessive gene [2,3]. Phenotypically, the mutation appears in homozygous embryos as a severe general deficiency of the skeletal musculature while cardiac and smooth muscles show normal development and function [2,3].

Extensive functional and molecular studies [4–8] showed that the probable gene defect that is the critical locus for the development of the dysgenic phenotype is located in or in close proximity to the skeletal muscle L-type voltage-gated calcium channel α_1 subunit gene (CACHL1A3). Apparently, normal level expression was found for the α_2/δ subunit (CACHL2A) [7]. Thus far, no studies have been conducted for detection of β subunit transcripts or proteins. In recent molecular genetic analyses, we assigned the CACHL1A3 gene as the *mdg* locus that resides on chromosome 1 either for human or for the mouse [9]. Cloning and sequencing studies of cDNAs to CACHL1A3 gene in the *mdg* mouse disclosed a consistently appearing point mutation, a cytosine deletion at nt 4010 that causes a frameshift and premature translation stop in the reading frame of the skeletal muscle L-VGCC α_1 transcript [10]. This observation, however, is contradictory to a previous analysis [8] with genomic DNA for the region of the CACHL1A3 gene, in which alterations were found in the restriction fragment length of the 5′-end and 5′-flanking region for the CACHL1A3 gene, providing evidence that the lack of expression or low level expression of the α_1 transcript is likely a consequence of a mutation in the promoter region of the gene.

A permanent, immortalized cell line was recently established from the *mdg* mouse [11] that retains the differentiation capability and all functional and phenotypical characteristics of the dysgenic mutation. Here, we present molecular evidence that the dysgenic mutation is fully retained in these cells as a point mutation in the CACHL1A3 gene at nt position 4010 of the open reading frame. We observed considerable expression of the CACHL1A3 gene transcripts as well as transcription of the class C-type (cardiac) CACHL1A1 calcium channel gene. The transcription of the latter gene can be either due to aberrant differentiation [12] in *mdg* cells or to a 'turn-on' of a compensatory mechanism that controls the level of calcium channel transcripts. Consistent with this finding, we detected class C-type calcium channel activity in these *mdg* cells.

2. Materials and methods

2.1. Cell cultures

The normal mouse muscle cell line ($129CB_3$) and the dysgenic muscle line ($129DA_3$) were grown at 34°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum and 1.25% chicken embryo extract. Differentiation was induced by deprivation of growth factors and shifting the temperature of growth to 39°C [11]. The differentiation medium contained 10% horse serum and 1.25% chicken embryo extract in DMEM. For routine growth of the cells, tissue culture dishes were coated with 1% gelatine.

2.2. RNA extraction, Northern blotting and hybridization

Total RNA was extracted from 10^7–10^8 cells by the acidic guanidinium thiocyanate method [13] and polyA$^+$ RNA was purified on oligo dT cellulose columns [14]. PolyA$^+$ RNA (2–5 μg) was denatured and size separated on 1.2% denaturing formaldehyde-agarose gels and transferred onto Hybond C filters (Amersham). Filters were hybridized with ^{32}P-labeled [15] DNA probes in 50% formamide, 0.5 M NaCl, 10 mM Na/PO_4, pH 7.4, 5 mM EDTA, 0.1% SDS and 10% dextrane sulphate at 45°C. The highest stringency washing of filters was done in 150 mM NaCl, 10 mM Na/PO_4, pH 7.4, 1 mM EDTA and 0.1% SDS at 55°C. Blots were exposed to Kodak X-OMAT films at −70°C for various times (from 2 to 24 h).

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Abbreviations: *mdg*, muscular dysgenesis; L-VGCC, L-type voltage-gated Ca^{2+} channel; nt, nucleotide.

SSDI 0014-5793(95)00697-4

2.3. PCR amplification of segments of skeletal and cardiac α_1 gene products

Multiplex PCR was performed on 2 μg polyA$^+$ RNA isolated from non-differentiated and differentiated cultured mouse muscle cells ($129CB_3$, $129DA_3$) and from murine muscle tissues. The first strand synthesis was done with 1 μg polyA$^+$ RNA using 200 U of Superscript reverse transcriptase (GIBCO-BRL) and 6-mer random primers at 50°C for 1 h. For the second strand synthesis and amplification, multiplex PCR was performed using 2 U of Vent polymerase and three primers. The reverse primer was designed for a region in motif I that is highly homologous between skeletal [10] and cardiac α_1 subunits [16]. This sequence lies between nts 1942–1962 and 675–695 for cardiac and skeletal muscle clones, respectively. The two forward primers were designed for a more 5′-upstream sequence that is non-homologous between the cardiac (1249–1268) and skeletal (206–226) messages. PCR (Roche Molecular Systems) was done in 32 cycles including denaturation at 95°C for 2 min, annealing at 68°C for 30 sec and extension for 2 min at 72°C. The PCR products were separated on 2% sieving agarose gels. RT-PCR amplification for the motif IVS_5-S_6 segment of both skeletal and cardiac α_1 was done utilizing the same conditions as described for motif I regions. The primers were designed for nts 3935–3954 (forward) and 4108–4087 (reverse) corresponding to the skeletal α_1 sequence. The PCR products were isolated from preparative gels, subcloned into pBluescriptSK(+) at the *Eco*RV site and sequenced using the dideoxy chain termination method [17].

2.4. Cell cultures for electrophysiology and electrophysiological recordings

Cells were plated at very low density (100–500 cells/60 mm dish) and grown on glass coverslips coated with 1% gelatin. 24 h after plating, differentiation was induced as described above and electrophysiological recordings were done at a very early stage of differentiation (usually on the second and third day of differentiation) when most of the cells were in the non-fused stage or myotubes did not contain more that 2–3 nuclei/cell. Barium currents (I_{Ba}) were recorded in the whole-cell configuration [18] at room temperature (20–22°C). The bathing solution was (in mM): $Ba(OH)_2$, 40; glutamate, 40; *N*-methyl-D-glucamine, 80; HEPES, 10; $MgCl_2$, 2; pH adjusted to 7.4 with CH_3SO_3H. The pipette solution contained (in mM): *N*-methyl-D-glucamine, 110; $MgCl_2$, 2; EGTA, 15; HEPES, 15; pH adjusted to 7.3 with CH_3SO_3H. Pipettes had resistances between 3–5 MΩ when filled with 1 M KCl. Capacitive transients were minimized using the analog circuitry of the amplifier (Axopatch 1B, Axon Instruments, CA). I_{Ba}s were recorded at various digitizing rates and filtered at 0.5 or 1 kHz using a four-pole Bessel filter. Stimulation of the cell, data acquisition and analysis were performed using the pCLAMP software package (version 5.5; Axon Instruments,CA).

3. Results

In Northern hybridizations, a skeletal muscle L-VGCC α_1 (α_{1S}) subunit probe provided clear signals with a 6.5-kb mRNA species for both of the differentiated $129CB_3$ and $129DA_3$ cells. The hybridizing RNA band was of identical size with that detectable in a mouse skeletal muscle preparation. When the same blot was hybridized with a cardiac L-VGCC α_1 (α_{1C}) probe, cross-hybridization was noted with the 6.5-kb band, however, an 8.6-kb band was clearly detectable in differentiated $129DA_3$ cells, the size of which was identical with cardiac α_1 mRNA from mouse heart (Fig. 1). A skeletal muscle α_2 probe detected hybridization with an 8.5-kb message for $129CB_3$ and $129DA_3$ cells as well. The skeletal muscle β_{1a} probe cross-hybridized with four bands corresponding to RNA sizes of 1.9, 3.5, 5.3 and 6.8 kb. These correspond to products of β_1, β_2, β_3 and β_4 genes. The pattern of expression of β messages in immortalized normal and dysgenic muscle cells is somewhat different from that seen in normal skeletal muscle. The immortalized cells express commensurable amounts of all four β messages while the β_1 gene product is prevalent in normal skeletal muscle

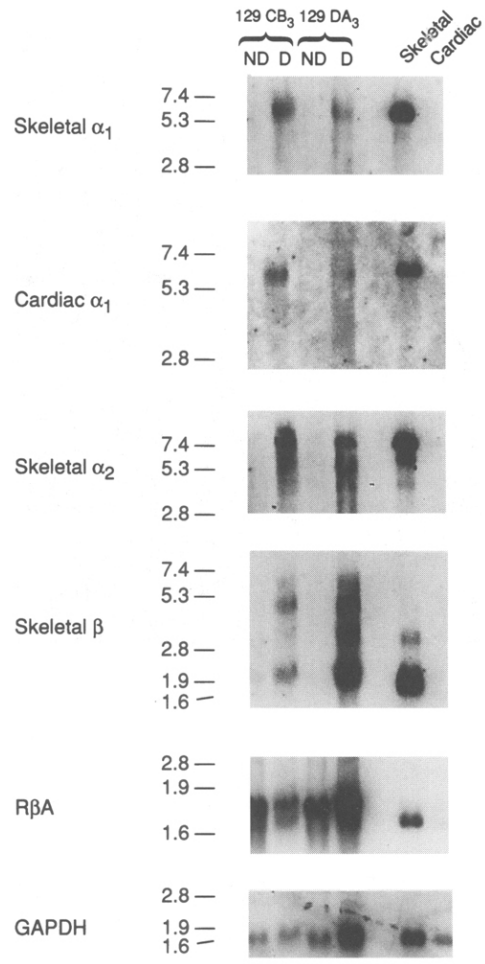
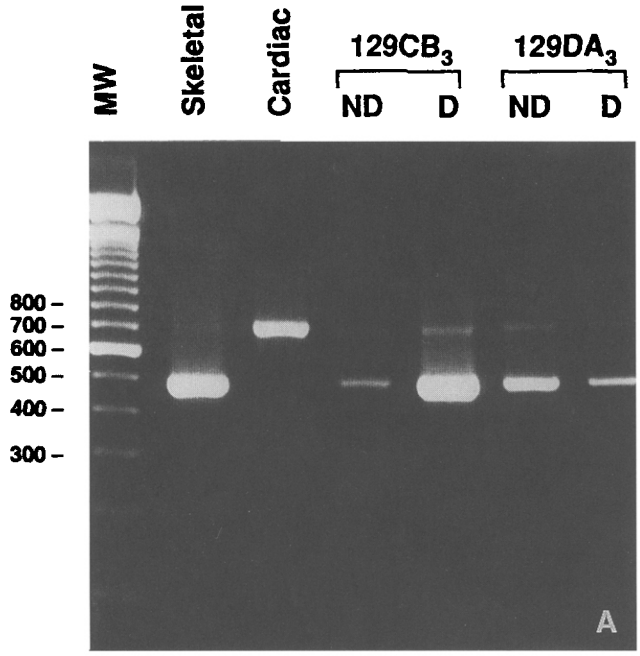


Fig. 1. Expression of mRNAs of L-VGCC subunits in normal ($129CB_3$) and dysgenic ($129DA_3$) mouse muscle cells. PolyA$^+$ RNAs from $129CB_3$ and $129DA_3$ cells were hybridized with various Ca^{2+} channel subunit cDNA probes. ND, non-differentiated; D, differentiated. The probes are: skeletal α_1; 6.1-kb *Xba*I fragment of the full-length rabbit cDNA [19], cardiac α_1; 5.2-kb *Eco*RI fragment of the rabbit cDNA [20], skeletal α_2; 3.5-kb *Eco*RI fragment of the rabbit cDNA [19], skeletal β; 1.6-kb *Eco*RI fragment of the rabbit cDNA [21], RβA; 1.6-kb *Pst*I fragment of the cross-hybridizing rat β-actin probe [23], GAPDH; 0.7-kb *Pst*I/*Xba*I fragment of the glyceraldehyde-3-phosphate dehydrogenase cDNA [22].

and the expression of β_2 and β_3 genes is significantly lower. The β_4 gene expression was not detectable in mouse skeletal muscle.

A cross-hybridizing β-actin gene cDNA was used to monitor the progress of differentiation. Hybridization with this probe shows that during differentiation the expression of α-actin is turned on, however, β,γ-actin expression is not completely eliminated. This phenomenon is typical for the immortalized muscle cells [23]. The expression of skeletal and cardiac α_1 transcrips were assessed relative to the level of the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene. We estimated that skeletal α_1 expression was 10–30-fold lower in differentiated $129DA_3$ cells compared to that of $129CB_3$ normal mouse muscle cells.

RT-PCR amplification of α_{1S}- and α_{1C}-specific cDNA se-



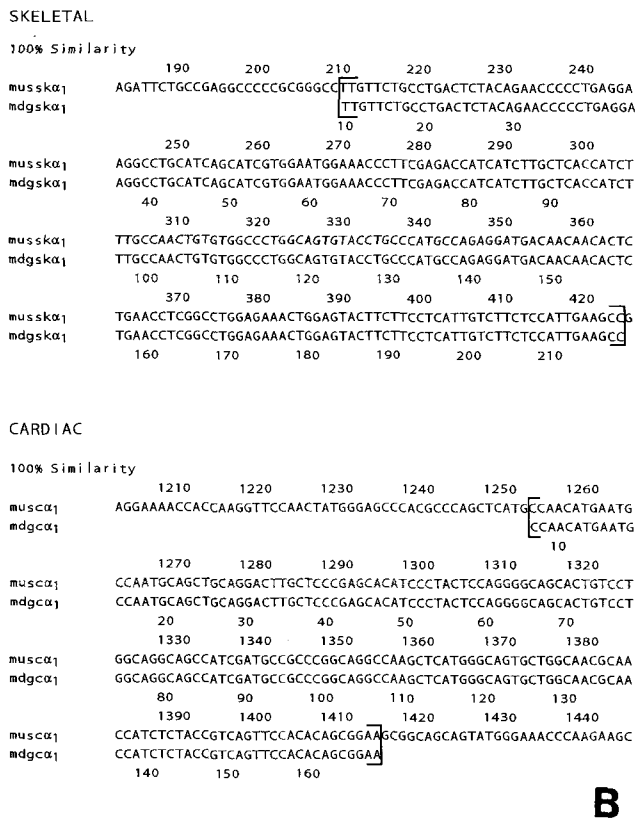


Fig. 2. Multiplex RT-PCR of the divergent 5'-ends of mouse skeletal and cardiac α_1 sequences from polyA$^+$ RNA, isolated from non-differentiated and differentiated cultured mouse muscle cells ($129CB_3$, $129DA_3$) and from muscle tissues. (A) Three primers were used for the PCR (see Section 2). Amplification between these primers provide reaction products of 694 and 470 nts for cardiac and skeletal α_1, respectively. (B) A region of 5'-amplified sequences are depicted and shown that they are in 100% homology with the corresponding skeletal and cardiac α_1 sequences.

quences was carried out on mRNA preparations from non-differentiated and differentiated forms of $129CB_3$ and $129DA_3$ cells. Multiplex PCR resulted in the appearance of 470 and 694 bp products in both cell lines that are characteristic cDNA segments for skeletal and cardiac α_1, respectively (Fig. 2A). The DNA sequences of the PCR products showed 100% identity with the mouse skeletal and cardiac α_1 sequences (Fig. 2B). The amplifications have not been optimized on quantitative scale, thus no conclusions can be reached for the relative abundance of α_{1S} and α_{1C} transcripts in these cells.

PCR amplification on a segment (IVS_5-IVS_6, between nts 3935–4087) resulted in the appearance of a product with 152 bp in length both for $129CB_3$ and $129DA_3$ mRNA preparations (data not shown). Sequencing of the $129DA_3$ mRNA related PCR product on both strands revealed a DNA sequence that was 100% identical with that of the mouse α_{1S} except at nt position 4010. At this position, a deletion mutation, the lack of a cytosine nucleoside residue, was noted compared to the sequence obtained from $129CB_3$ cells and matched that published previously [10] (Fig. 3A). The existence and nature of this mutation was confirmed by using several different mRNA preparations from $129CB_3$ and $129DA_3$ cells and also by independent amplifications of the same RNA preparation. We have found that the deletion mutation consistently appears in $129DA_3$ cells and never appears in the normal counterpart, in $129CB_3$ cells. The mouse α_{1C} sequence for this region did not show this mutation and was found to be fully identical with the published mouse cardiac α_1 sequence (data not shown) [16].

The cytosine deletion at nt 4010 in the $129DA_3$ α_1 sequence generates a frameshift mutation in the conceptual translation. This frameshift generates a stop codon at nt 4173 that prematurely terminates the translation of the α_1 message. Consequently, part of the topological arrangement of the α_1 in motif IV is altered. The translation stops in the mutant *mdg* α_1 after the motif IV SS1 region and the critical SS2 segment that carries glutamic acid residues important for Ca^{2+} selectivity are missing [24–26]. Further, the IVS_6 and the C-terminal tail are deleted (Fig. 3B).

Depolarization of voltage-clamped $129DA_3$ cells from a holding potential of −80 mV to various test potentials from −40 to +50 mV induced inward barium currents (I_{Ba}) in a voltage-dependent manner (Fig. 4A). The currents activated with fast kinetics and decayed following inactivation kinetics, in a manner typical for cardiac L-type I_{Ba}s. The current-voltage relationship of these current recordings is depicted in Fig. 4B. The currents activated at −30 mV and reached peak current values at +10 mV potential. This high-voltage activated I_{Ba} was found to be sensitive to dihydropyridine agonists. Application of 1 μM Bay K 8644 increased the peak current ~4-fold, shifted the voltage-dependence of activation and inactivation to hyperpolarizing potentials and accelerated inactivation (Fig. 4C). The I_{Ba}s also exhibited sensitivity to β-adrenergic modulation. Isoproterenol at a concentration of 10 μM increased I_{Ba} ~2-fold (Fig. 4D). In our experiments, we never detected slow L-type I_{Ba} that would be related to skeletal L-type Ca^{2+} channel activity.

4. Discussion

We have shown that the *mdg* mutation is molecularly retained in an immortalized cell line ($129DA_3$) and is in the α_1

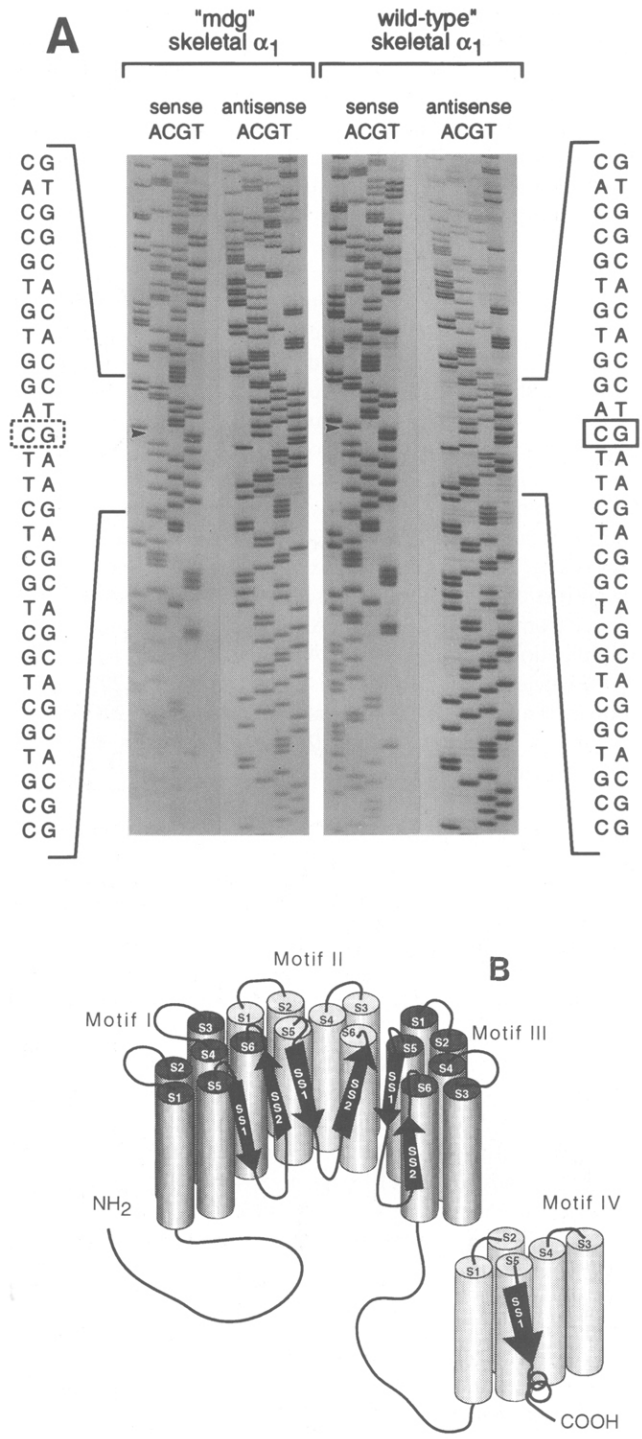


Fig. 3. (A) RT-PCR amplification of skeletal α_1 sequences from polyA$^+$ RNA isolated from differentiated 129CB$_3$ and 129DA$_3$ cells. The primers were designed for nts 3935–3954 (forward) and 4108–4087 (reverse) that spans the region between IVS$_5$ and IVS$_6$ and covers the SS1-SS2 segments. The PCR products were subcloned and sequenced. Some details of the sequence are depicted on the figure. In PCR products from *mdg* cells (129DA$_3$) a point mutation, a deletion of a cytosine residue was noted at nt 4010 (left, the missing CG base-pair was put in the box with dashed lines). In PCR products of normal mouse cells (129CB$_3$), no cytosine deletion was detected (right, the base pair at 4010 is boxed). (B) The deletion mutation at nt 4010 in dysgenic mouse cells introduces a frameshift mutation into the skeletal muscle isoform of the calcium channel α_1 subunit cDNA. Due to the frameshift, a stop codon is introduced at nt 4173 that prematurely terminates the translation of mRNA and changes part of the topology of motif IV as well. ←

subunit of L-VGCC at nt 4010 where a cytosine residue is deleted. This deletion generates a frameshift that culminates at a premature stop codon at nt 4173. Previously, we showed for the genome of the *mdg* mouse that the genetic locus of the mutation is colocalized with the L-VGCC α_1 subunit gene [9]. Further, cDNA cloning and sequencing of the *mdg* mouse L-VGCC α_1 also identified the nature and position of *mdg* point mutation [10].

The *mdg* point mutation has widespread consequences for the nature and function of the skeletal muscle L-VGCC α_1 subunit. First, due to the presence of the premature stop codon, the mutant transcript codes for a protein with a length of 1352 amino acids and a molecular weight of 153,681. This is a considerable truncation compared to that of the full-length sequence of the α_1 from normal mouse muscle (1873 amino acids, molecular weight 212,018). Second, in the mutant protein part of the IVSS1, the IVSS2, the extracellular connecting loop to IVS$_6$, the IVS$_6$ and the entire C-terminal cytoplasmic tail is missing. Some of these segments, in particular the IVSS1 and IVSS2, are well-identified integral components of the high-affinity Ca^{2+} filtration units [24–26]; the putative E-F hand [27] on the C-terminal tail may also be involved in Ca^{2+} binding. Based on hydropathy analysis and prediction of secondary structure, we suggest that the C-terminus of the mutant protein will be largely globular and localized intracellularly. Therefore, it is unlikely that the mutant α_1 protein will and can exert calcium channel function. Our electrophysiological data are consistent with this since no skeletal L-type I_{Ba} was recorded in dysgenic 129DA$_3$ cells [11].

Immortalized dysgenic mouse skeletal muscle cells express detectable amounts of skeletal and cardiac L-VGCC α_1 subunit mRNAs. The expression level for the skeletal α_1 transcript was reported to be dramatically diminished compared to that obtained in normal mouse skeletal muscle cells [10]. We cannot understand, however, how the single point mutation around the middle of the mRNA could be responsible for the reduced mRNA levels [10]. Most likely, the decreased expression is due to some compensatory mechanisms activated by the altered Ca^{2+} homeostasis of these cells. Alternatively, decreased mRNA stability is also possible [10,12] although it is difficult to understand the underlying molecular mechanisms. The present and previous data [10,12] are contradictory to an earlier description that localized the mutation of muscular dysgenesis in the 5′-end and flanking sequences of the skeletal muscle L-VGCC α_1 subunit gene [8]. Two other skeletal muscle L-VGCC subunit mRNAs; two transcripts for α_2/δ and numerous β subunit transcripts are expressed at a level similar to that observed in control cells. Further, this provides an explanation as to why expression of skeletal [8], cardiac [28] and brain [29] α_1 cDNAs in primary cultures of *mdg* muscle cells always elicited Ca^{2+} channel currents that were very close to the native behavior.

It is tempting to speculate about possible phenotypic characteristic of dysgenic skeletal muscle in view of the causative point mutation and the present discovery of cardiac Ca^{2+} channels.

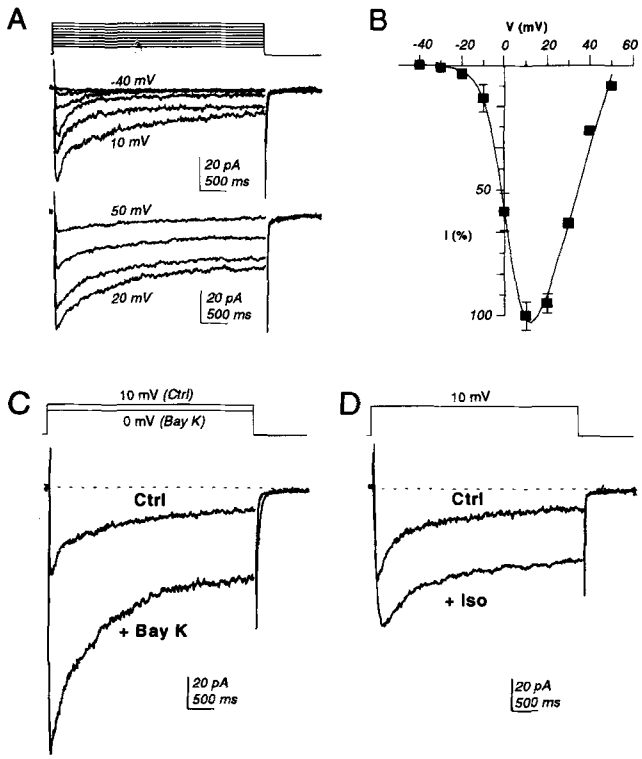


Fig. 4. Electrophysiological properties of Ca^{2+} currents endogenously expressed in dysgenic muscle cells. (A) Current traces were recorded from −20 to +50 mV (HP was −80 mV). (B) Current-voltage relationship of I_{Ba}. Numerous recordings are normalized and the percentage of peak current is depicted against membrane potential. (C) Influence of a calcium channel agonist Bay K 8644 on I_{Ba}. (D) β-adrenergic stimulus enhances the calcium channel activity in $129DA_3$ cells.

It is conceivable that the lack of excitation–contraction (E–C) coupling is due not only to the low level of aberrant α_{1S} subunits but also to a process that excludes the endogenous α_{1C} from interacting with the coupling machinery. Interestingly, overexpression of cardiac Ca^{2+} channels restores a modified, cardiac-like E–C coupling [28] while overexpression of the α_{1A} [29] or α_{1B} [30] cDNAs, which deliver sizable Ca^{2+} currents, fail to restore E–C coupling.

Our data provide evidence that α_{1C} is overexpressed in the $129DA_3$ dysgenic cell line, compared to the normal $129CB_3$ counterpart. This result is reminiscent to that observed in developing skeletal muscle [12]. Consistent with the presence of the α_{1C} transcript, we routinely recorded cardiac L-type current in differentiating myotubes of $129DA_3$ cells. Here, we have demonstrated that the I_{Ba} recorded in dysgenic myotubes reflect the activity of α_{1C}-directed Ca^{2+} channels whose electrophysiological, pharmacological and β-adrenergic regulation properties are the same as those of the class C-type Ca^{2+} channels [31]. This is the first experimental evidence showing that the appearance of α_{1C} transcript in *mdg* cells gives rise to functional, C-type Ca^{2+} channel activity. While it is possible that the level of expression of the endogenous α_{1C}, as well as the corresponding Ca^{2+} channel current may be unable to provide enough 'Ca^{2+} entry' to restore E–C coupling in the dysgenic cell line [11,32], this is unlikely since, the amount of 'trigger Ca^{2+}' required to elicit a contraction is extremely small [33,34]. We therefore suspect that some component required for E–C coupling is missing. On the other hand Tanabe et al. [28] demonstrated that overexpression of α_{1C} in dysgenic myotubes can restore C-type E–C coupling. The presence of cardiac L-type Ca^{2+} channel in *mdg* myotubes is probably related to the etiology of the disorder [35] and is compensatory in nature. Thus, the expression of cardiac α_{1C} in $129DA_3$ cells would be reminiscent of the early stages of skeletal muscle development.

Acknowledgments: This work was supported by NIH Grants HL 22619–17, HL 43321–7 and by a grant from Tanabe Seiyaku Co. Ltd. Gabor Mikala is a Research Associate of the Program of Excellence in the Molecular Biology of Heart and Lung (HL 41496–07). The authors thank to Ms. Gwen Kraft for preparing the figures.

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